Effects of Inhaled Alpha-Emitting Actinides on Mouse Alveolar Macrophages

by Arthur Morgan[1] and Robert J. Talbot[1]

The effects of inhaled alpha-emitting actinides on the alveolar macrophage (AM) population of the rodent lung are reviewed and, in particular, of the effects of ^{239}PuO$_2$ on murine AM. The effects discussed include changes the AM pool size, macrophage diameter, mobility, phagocytic competence, and enzyme content. Finally, similarities in the dose–response relationships for the induction of nuclear aberrations by alpha emitters and in the induction of lung tumors by the same materials are noted.

Introduction

Because alveolar macrophages can be recovered from the lungs of experimental animals in large numbers, this is a particularly convenient cell with which to monitor the effects of radiation on the lung. Although the alveolar macrophage is not thought to be a tumor precursor cell per se, it secretes a wide variety of mediators including various enzymes, interleukin-1, tumor necrosis factor, and leukotrienes. The interaction of dust particles with macrophages causes the release of abnormal quantities of these products, which have the potential to react with and damage epithelial cells. Some of the effects of high doses of radiation mimic those induced by mineral dusts, so it seems likely that common factors are involved. At lower doses, however, the effects of radiation are more subtle because they affect the nuclear rather than the cytoplasmic contents of cells.

The inhalation of radioactive materials can result in the induction of lung tumors, and, in the mouse, the maximum tumor incidence after exposure to ^{239}PuO$_2$ corresponds to an initial alveolar deposit (IAD) of about 200 Bq. With greater IADs, there is a decline in tumor incidence but an increase in the severity of lung fibrosis (*1*), together with significant life shortening. At the other end of the dose scale, it is possible to detect significant increases in the incidence of alveolar macrophages with nuclear aberrations with IADs as low as 1 Bq. Thus, the range of doses of interest extends over three orders of magnitude.

Alveolar macrophages recovered from the lungs of experimental animals that have been exposed to relatively high levels of sparingly soluble compounds of alpha-emitting nuclides such as ^{239}PuO$_2$ show a number of changes compared to cells from unexposed controls. Some of these changes, for example, the presence of abnormally large cells and cells with bizarre nuclear aber-

rations, are obvious, but others are more subtle and less readily quantified. It is the purpose of this paper to review these changes. Most of the results discussed have been obtained in our own studies using the mouse, but, where appropriate, relevant information using macrophages of other species is included.

Effects on Macrophage Numbers

Moores et al. (*2*) have shown that there is a significant depression in the total number of alveolar macrophages (AM) following exposure to ^{239}PuO$_2$ to give IADs greater than 20 Bq. The lungs of the CBA strain of mouse used in our own studies contain about 1.7×10^6 AM, of which only about 25% can be recovered by bronchoalveolar lavage (BAL) *in situ*. The efficiency of lavage is variable, so that the number of AM recovered by BAL is a rather poor guide to the total pool size. It is possible to score the number of AM per alveolus in histological lung sections (*2*) to give an index of changes in pool size, but this is a tedious procedure. As a simpler alternative, we have developed a technique for labeling the AM pool with a sparingly soluble gamma-emitting tracer such as ^{169}Yb$_2$O$_3$. This tracer is normally administered by inhalation a few days before the animals are killed. The number of AM recovered by BAL can then be measured, along with their associated radioactivity, so the mean specific activity of AM can be calculated. If the activity remaining in the lung after BAL can be ascribed to the number of unlavaged AM, the size of the total AM pool can be estimated.

Most of the early studies of the effects of alpha emitters on the numbers of AM were relatively short term. It was shown that with low IADs (< 150 Bq), after a rapid decline in cell numbers that reached a nadir at about 2 weeks after exposure, control levels were reestablished after about 1 month. With greater IADs (150–500 Bq), the depression in numbers was even more marked, longer in duration, and followed by a chronic depression relative to controls. As the IAD is increased further into the "fibrotic" dose range (> 500 Bq), the initial fall and recovery in numbers is followed by an overshoot, and AM numbers remain permanently elevated (Fig. 1). It should be noted that abnormally

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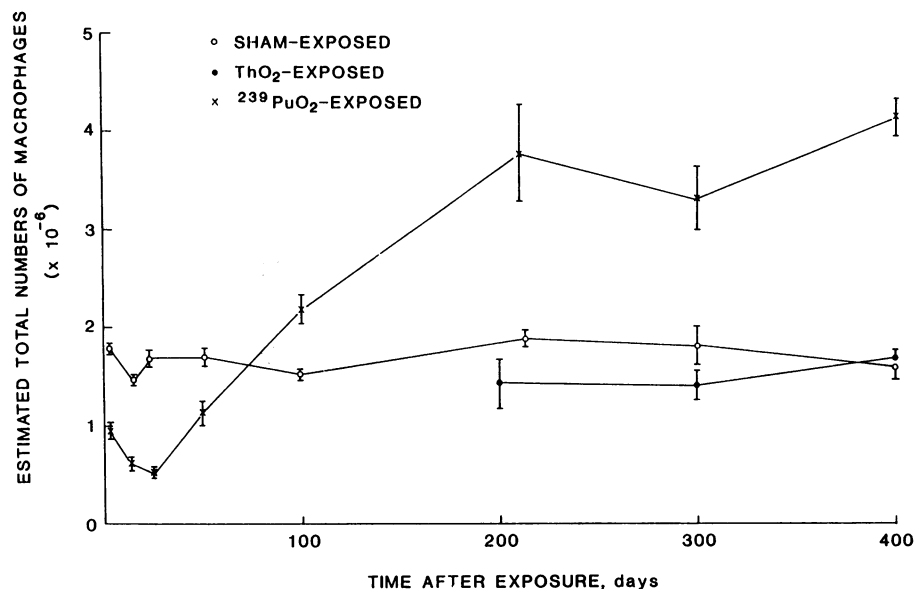
FIGURE 1.   Estimated total numbers of alveolar macrophages in the lungs of CBA mice after exposure to $^{239}PuO_2$ to give an initial alveolar deposit of 1100 Bq. Some
data for mice exposed to ThO_2 to give a similar mass deposit are included.

high numbers of AM also result from exposure to fibrogenic mineral dusts such as quartz (3), so this effect is not necessarily a consequence of exposure to radiation per se.

The relative effects of equivalent radiation doses from $^{238}PuO_2$, $^{239}PuO_2$, and $^{241}Am(NO_3)_3$ on the AM pool have been compared by Talbot et al. (4), who found that the depression was greatest with ^{241}Am, which gives the most uniform dose to lung, and least with ^{238}Pu, which gives the most heterogeneous dose. The cellular kinetics of the AM pool and possible reasons for the observed changes in cell numbers are discussed elsewhere in this issue (5).

Influx of Other Cell Types

The numbers of neutrophils and lymphocytes recovered from the lungs of control CBA mice by BAL are generally of the order of 1–2 × 10³ or 1% of all recovered cells. Their numbers only increase significantly following the administration of fibrotic doses of $^{239}PuO_2$. Eosinophils only occur in BAL from mice that already contains high numbers of polymorphonuclear leukocytes. Whether the influx of neutrophils occurs in response to the cytolysis products of AM or to the release of specific chemoattractants is not known.

Effect on Macrophage Size

Marked changes in the diameters of AM only become apparent with IADs exceeding 100 Bq. During the initial postexposure period, when cell numbers are declining, the mean size of lavaged AM increases rapidly, but when the AM pool enters the recovery phase, the influx of near-normal size AM into the alveolar spaces reduces the mean diameter of recovered cells. As shown in Figure 2, after a fibrotic dose of $^{239}PuO_2$, the median diameter of AM measured with a Coulter counter increased from a normal value of about 11.2 μm to a maximum of about 12 μm after 21 days. In the early stages of the recovery phase there was

a decrease in cell diameter, but this was followed by a further increase to give an even greater median diameter at all times after 200 days. This increase in diameter does not reflect a uniform increase in cell size, but is due rather to an increased frequency of very large cells with diameters greater than 14 μm. Although this increase in cell size may not appear great, it should be noted that macrophages with a diameter of 14 μm have twice the volume of normal cells.

Effect on Macrophage Mobility

The effect of exposure to $^{239}PuO_2$ on the mobility of macrophages recovered from rabbits at different times after exposure has been studied by Nolibe (6). Lavaged cells were collected in polyethylene capillary tubes, which were placed in Medium 199 containing autologous serum and incubated at 37°C for 24 hr. The areas covered by the macrophages migrating from the ends of the tubes were taken as a measure of their mobility. It was apparent that the mobility of AM recovered 2 days after exposure to ^{239}Pu was already less than that of cells from controls and, after 21 days, mobility was even further reduced.

In a similar study of migration (7), Hahn and Muggenburg recovered alveolar macrophages from dogs that had been exposed either to fused aluminosilicate particles (FAP) containing ^{144}Ce or to $^{239}PuO_2$. The AM were collected in capillary tubes, which were placed in chambers containing Medium TC199 with fetal calf serum. The migration of AM recovered from the dogs exposed $^{239}PuO_2$ was inhibited at all times following exposure, but no effect was observed with FAP containing ^{144}Ce.

Effect on Phagocytosis

The phagocytosis of particles of $^{239}PuO_2$ by macrophages was first studied by Sanders and Adee (8), who lavaged the lungs of rats exposed to an aerosol of this material. They found that some of the particles were already phagocytized by AM during the first
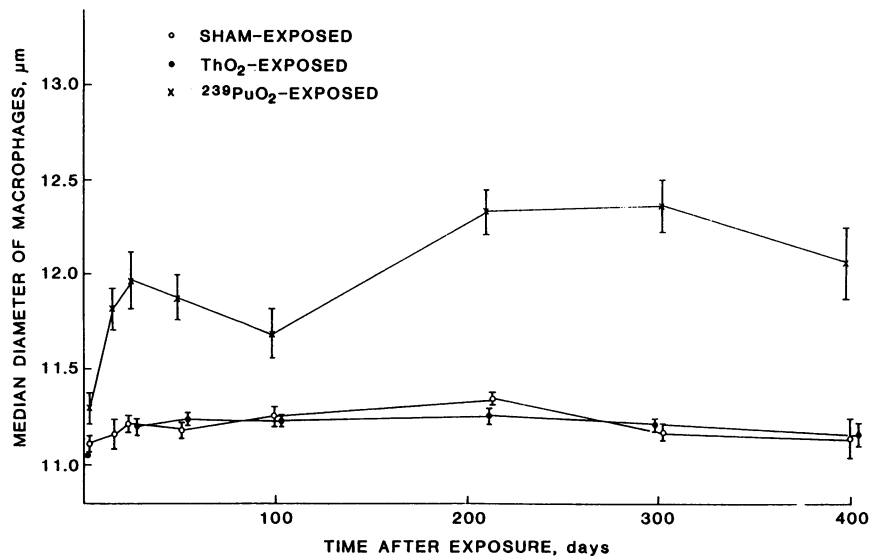
FIGURE 2. Median diameters of alveolar macrophages recovered by bronchoalveolar lavage from the lungs of mice exposed to $^{239}PuO_2$ to give an initial deposit of 1100 Bq. Data for mice exposed to ThO_2 to give a similar mass are included.

3 hr after exposure and retained within these cells for at least 25 days. By 2 days nearly all the deposited particles were within AM. Similar studies using rabbits were reported by Nolibe and Masse (9), who showed, after inhalation exposure, that 50% of the particles of $^{239}PuO_2$ were already phagocytized after 1 hr, and the uptake process was essentially complete by 24 hr. The survival times in vitro of AM recovered from ^{239}Pu-exposed rabbits was less than for cells recovered from controls.

More recently, the phagocytic competence of AM after exposure to alpha emitters has been investigated by Morgan et al. (10). Mice were exposed to $^{239}PuO_2$, and then on three subsequent occasions (5, 20, and 33 days after the original exposure), groups were exposed to an aerosol of fluorescent polystyrene microspheres (FPM) with diameter 1.1 μm. The distribution of FPM in randomly selected macrophages was determined and, following autoradiography, the ^{239}Pu contents of the same cells were measured. Contrary to expectation, Morgan et al. found that the cells that contained high levels of ^{239}Pu also contained large numbers of FPM. This association could be explained, either by assuming that the effect of the radioactivity is to activate the cells, or that both $^{239}PuO_2$ and FPM are deposited preferentially at the same sites in the lung and are phagocytized by the same fraction of the AM population. Whichever explanation is correct, it is clear that the phagocytic competence of macrophages that contain ^{239}Pu is in no way impaired and may even be enhanced relative to uncontaminated cells.

Effect on Cytoplasmic and Lysosomal Enzymes

The mean levels of lactate dehydrogenase (LDH) in AM recovered from mice exposed to $^{239}PuO_2$ to give an IAD of 1100 Bq are shown in Figure 3. LDH is found in the cytoplasm of both leukocytes and red blood cells and is also present in plasma. As

contaminating erythrocytes were only rarely observed in lavage fluid, the results in Figure 3 are thought to be a true measure of the activity of this enzyme in macrophages. During the first 100 days after exposure, the mean LDH activity mirrored changes in cell diameter and may simply reflect the increase in cytoplasmic volume. After 100 days, however, the increase in AM diameter was not matched by their LDH activity, indicating that the large "foamy" macrophages present at later times do not have a correspondingly large LDH activity.

The lysosomal enzyme β-glucuronidase was also measured in AM (Fig. 4). The measurement of β-glucuronidase activity in lavaged cells is much more sensitive than the LDH assay and uncomplicated by the possibility of enzyme leakage from the pulmonary vasculature. In cells recovered from control mice, the activity of this enzyme averaged about 1.4 mU/10^{-6} cells. As with LDH, the mean cellular β-glucuronidase activity peaked at 14 days when it reached about 3.3 mU 10^{-6} cells; after 100 days the mean β-glucuronidase activity remained elevated relative to controls but did not approach the high values observed in the early stages of the study. In Figure 4, values are also given for the enzyme content of AM recovered from mice that had inhaled ThO_2 to give a similar mass deposition to that of $^{239}PuO_2$. This shows that the observed effects can be attributed to the radioactivity of the ^{239}Pu and not to its chemical toxicity.

Mean levels of β-glucuronidase measured by Talbot et al. (4) in AM of the same strain of mice following administration of $^{238}PuO_2$, $^{239}PuO_2$, and $^{241}Am(NO_3)_3$. The IAD for ^{239}Pu was lower (580 Bq) than in the study described above, and the maximum enzyme activity (2.9 mU/10^{-6} cells) occurred later in time. The β-glucuronidase activity was much greater following exposure to ^{241}Am, reaching about 6.5 mU/10^{-6} cells after 35 days. Autoradiographic measurements showed that, in the early stages of this study, virutally all AM contained ^{241}Am activity: with ^{239}Pu only about half the cells were labeled and an even
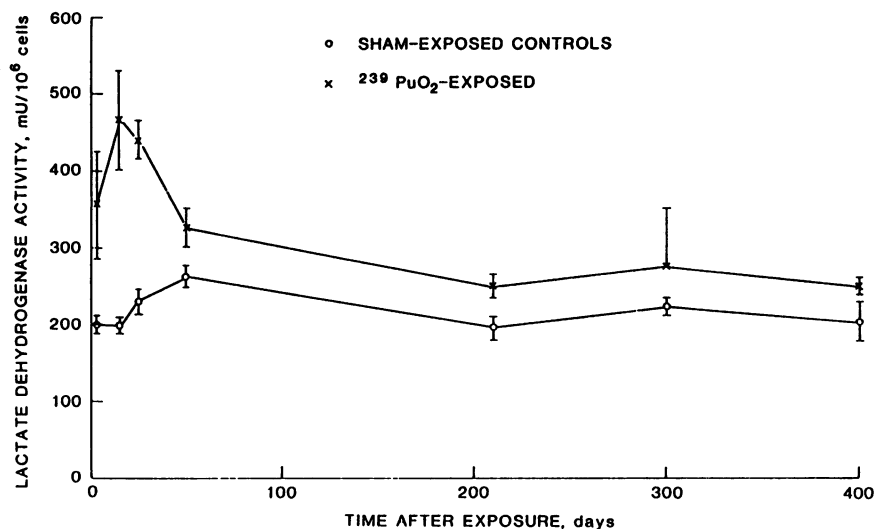
FIGURE 3. Lactate dehydrogenase activity in alveolar macrophages recovered by bronchoalveolar lavage from the lungs of mice exposed to $^{239}PuO_2$ to give an initial alveolar deposit of 1100 Bq.

FIGURE 4. β-Glucuronidase activity in alveolar macrophages recovered by bronchoalveolar lavage from the lungs of mice exposed to $^{239}PuO_2$ to give an initial alveolar deposit of 1100 Bq, from mice exposed to ThO_2, and from sham-exposed mice.
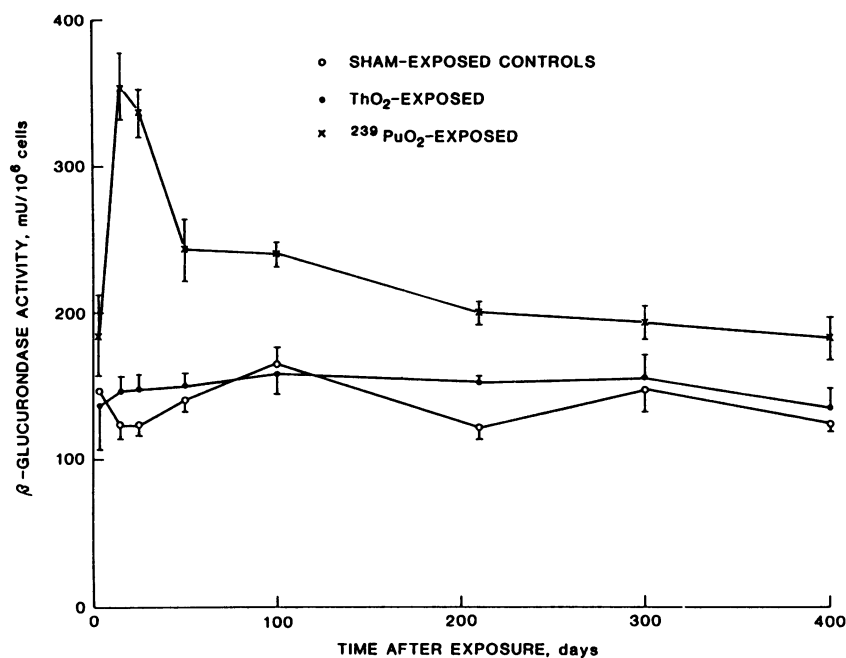
smaller proportion with ^{238}Pu. Clearly, the more uniform the radiation dose to lung, the greater the effect on cell enzyme levels.

As part of the same study, the β-glucuronidase activity of individual AM was measured using enzyme histochemistry after exposure to $^{239}PuO_2$. Cytocentrifuge slides were prepared and the cells stained with naphthol AS-BI glucuronide and hexazonium pararosaniline using a simultaneous coupling technique

(11). An image-analysis system was used to measure the areas of at least 250 AM and their optical densities. This enabled an integrated optical density (the sum of the number of pixels multiplied by their individual grey levels) to be determined for each cell. The image analyzer was used in conjunction with a scanning stage so that the coordinates of all measured cells were stored. Subsequently, the cytocentrifuge sliides were prepared for autoradiographic assessment using a stripping film (Kodak

AR10) technique. Cells, the optical density of which had been measured previously, were relocated automatically in the center of the microscope field and the number of associated alpha tracks determined. The cells were classified into the following track categories: 0, 1, 2–5, 6–20, 21–50 or > 50 tracks. The integrated optical densities (IODs) of macrophages recovered in lung washes 1 and 2 and 3–10 from an unexposed control mouse are shown in Figure 5A, and corresponding data for cells from a ^{239}Pu-exposed mouse, killed at 25 days after exposure are shown in Figure 5B. It can be seen that a preponderance of larger cells were recovered in washes 1 and 2 and smaller cells in the subsequent washes. Although in the ^{239}Pu-exposed mice, the IODs of most of the large cells fell on an extension of the relationship between IOD and cell area defined by the control, there were a number of cells present with anomalous IODs that were mostly recovered in washes 1 and 2. It appears that these large cells are recovered more readily by lavage, either because they are more easily detached from the alveolar surface or because they are located in a region of the lung that is more accessible to the lavage fluid.
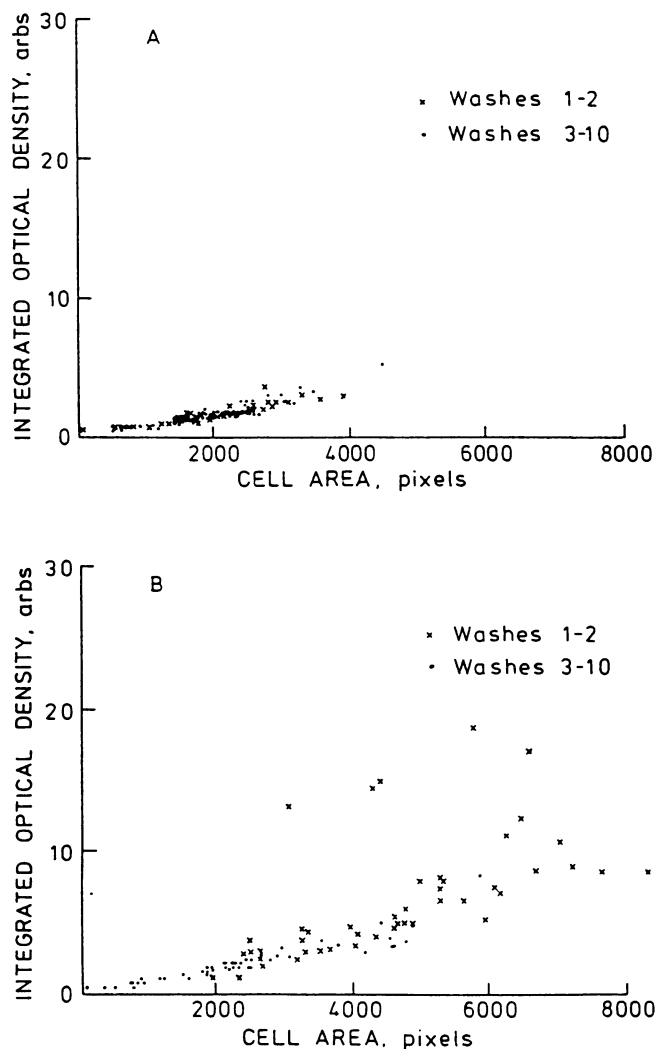

Measurements of β-glucuronidase in AM from unexposed control mice showed that the activity was distributed normally with a mean IOD of about 1.5 arbitrary units. As shown in Figure 6, the enzyme contents of AM from mice exposed to ^{239}Pu were distributed log-normally with median values of about 7, 4, and 3 arbitrary units at 25, 210, and 400 days, respectively. The geometric standard deviations were greater at the later time points than at 25 days due to the presence of significant numbers of AM with very high enzyme levels—up to 50 arbitrary units.

From Figure 7, it is apparent that the β-glucuronidase contents of AM were correlated positively with their associated ^{239}Pu activities at all time points. It is also clear that the IODs of cells without any associated alpha tracks were elevated relative to AM from control mice, indicating an enhanced enzyme content. In this study, the mean ^{239}Pu content of AM fell from about 1 mBq per cell at 25 days to 0.01 mBq per cell at 400 days, so the radiation dose to ^{239}Pu-free cells from cells containing activity will have decreased considerably during the study.

Increased enzyme levels may affect the physiology of AM. For example, Talbot et al. (12) has shown that when radioactive ^{169}Yb$_2$O$_3$ was administered to mice by inhalation followed by ^{239}PuO$_2$, the ^{169}Yb dissolved more rapidly in animals exposed to ^{239}PuO$_2$ than in those exposed to ^{169}Yb$_2$O$_3$ alone. This difference could be due to changes in the intraphagolysosomal pH of AM in mice exposed to ^{239}PuO$_2$.

Induction of Nuclear Aberrations

The effects of alpha emitters on the alveolar macrophage discussed so far are only readily measured at doses of at least 1 Gy. There are more sensitive indices of exposure, however, such as the induction of nuclear aberrations. As discussed elsewhere in this issue (5), it is now thought that, under normal circumstances, the AM population of the rodent lung is largely self-sustaining by division of AM *in situ* although there may be a contribution from monocyte-type precursors. Both these cells will be irradiated by alpha emitters deposited in the lung, and the dose to AM will be greater than to any other cell type.

The effect of this radiation on the macrophage can either be lethal, leading to cell death and cytolysis, or sublethal, in which case the cell may continue to function but be incapable of division. The decrease in macrophage numbers observed after the deposition of alpha emitters in the lung is due to a combination of cell death and an arrest of the normal cell cycle, so cells leaving the lung via the mucociliary escalator are not replaced. In the CBA mouse, at least 95% of AM recovered by lavage are in the G_1 phase of the cell cycle, and less than 0.1% have mitotic figures. Because the incidence of cells in mitosis is so low, it is difficult to demonstrate any change with statistical certainty.

With cells that are damaged sublethally but can still divide, radiation damage is expressed in the form of nuclear aberrations. These include binucleate AM (produced when the nucleus of a cell dividies but where their is a failure of cytokinesis) and micronucleate AM (where acentric fragments of chromosomes fail to be incorporated into the daughter nuclei during cell division). Cells with both types of aberrations may also be encountered, and; particularly at high doses, bizarre nuclear configurations are often seen in which two nuclei within the same cell are connected by a bridge of nuclear material.
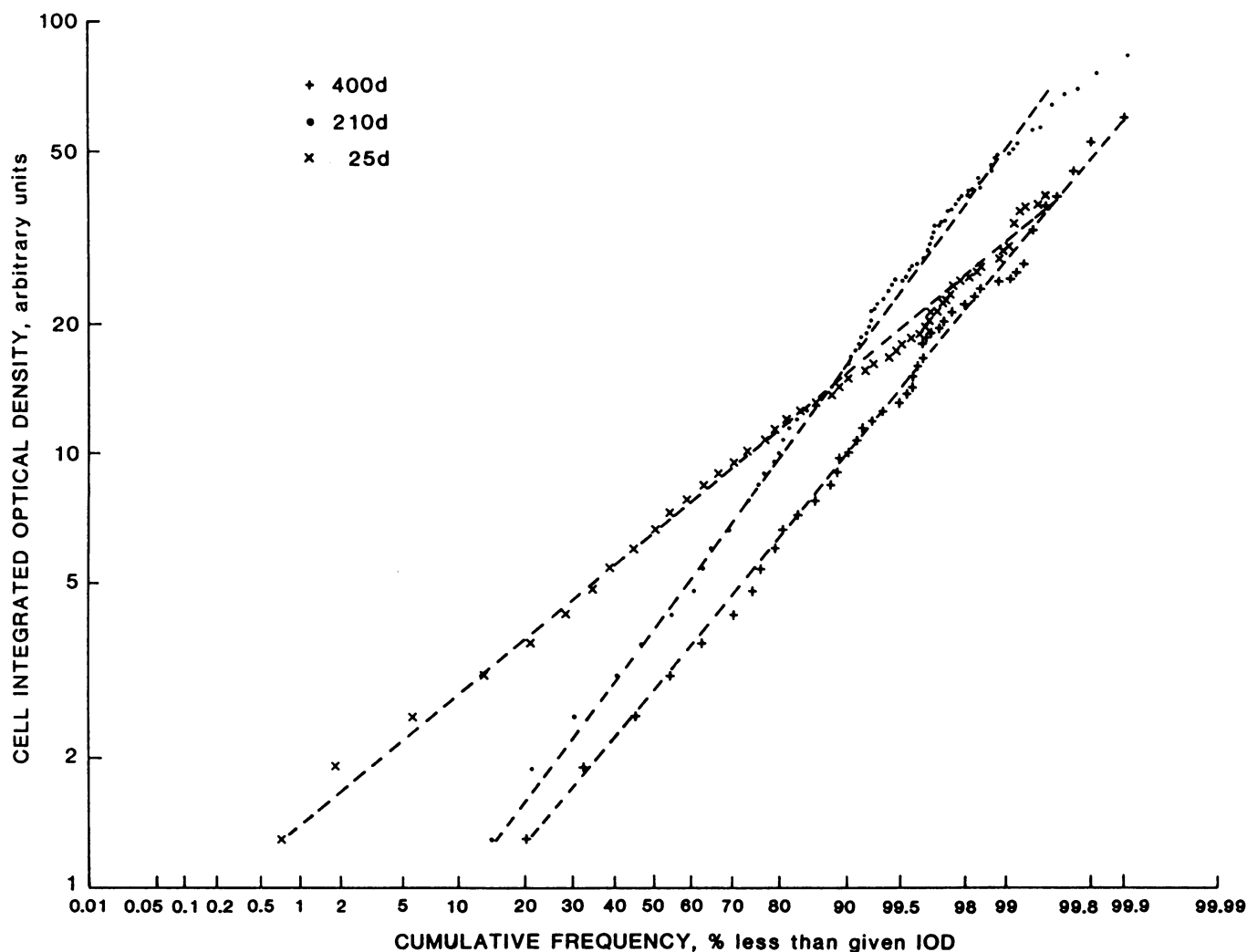
FIGURE 5. Integrated optical density (β-glucuronidase activity) versus cell area of alveolar macrophages recovered by bronchoalveolar lavage from the lungs of (A) sham-exposed mice and (B) mice exposed to ^{239}PuO$_2$ to give an initial alveolar deposit of 1100 Bq.

FIGURE 6.   Distribution of integrated optical density (β-glucuronidase activity) of alveolar macrophages recovered by bronchoalveolar lavage from the lungs of mice at 25, 210, and 400 days after exposure to ^{239}PuO$_2$ to give an initial alveolar deposit of 1100 Bq.

Examination of AM recovered from mice exposed to insoluble alpha-emitting nuclides shows that, although the cells decrease in number and increase in size, no nuclear changes are seen until the start of the recovery phase, which may be from 2 to 3 weeks after exposure. During this "latent" period, the AM cell cycle is arrested, and the incidence of nuclear aberrations only starts to increase when this block is removed and radiation damage can be expressed. The incidence of AM with micronuclei (MiAM), with two nuclei (BiAM), and with both types of aberration increases rapidly to a peak which, at lower radiation doses coincides with the restoration of the AM pool to its normal size. Subsequently, the incidence of MiAM decreases more rapidly than that of BiAM, indicating that the former may be shorter lived.

Using autoradiographic methods, we measured the distribution of alpha tracks in normal and aberrant AM from mice exposed to ^{239}PuO$_2$ (13). The track distribution patterns were identical in normal AM and in BiAM, indicating that the cells from which the latter were derived had normal phagocytic properties. MiAM generally contained less activity, which is to be expected as the

^{239}Pu content of the parent cell is shared, not necessarily equally, between the daughter cells. AM with mitotic figures generally contained little or no ^{239}Pu.

Exposure of rodents to chemical agents such as cigarette smoke (14) also produces a marked increase in the incidence of BiAM, which are formed by the failure of cytokinesis. Thus, it appears that MiAM are better indicators of radiation damage, and they also have the advantage that their spontaneous incidence is zero for all practical purposes, while, in the CBA mouse, about 0.3% of AM are binucleate. A significant increase in the incidence of MiAM can be detected with IADs as low as 1 Bq, which corresponds to a cumulative radiation dose to lung of only 50 mGy.

Because cell numbers change rapidly after exposure to alpha emitters, the incidence of AM with aberrant nuclei is not a particularly satisfactory index of radiation damage. However, using the ^{169}Yb-labeling technique described earlier, it is possible to estimate the total numbers of the various types of aberrant cells. In Figure 8, the estimated total numbers of MiAM in the lungs of CBA mice at 35 days after exposure to ^{239}PuO$_2$ is shown ver-
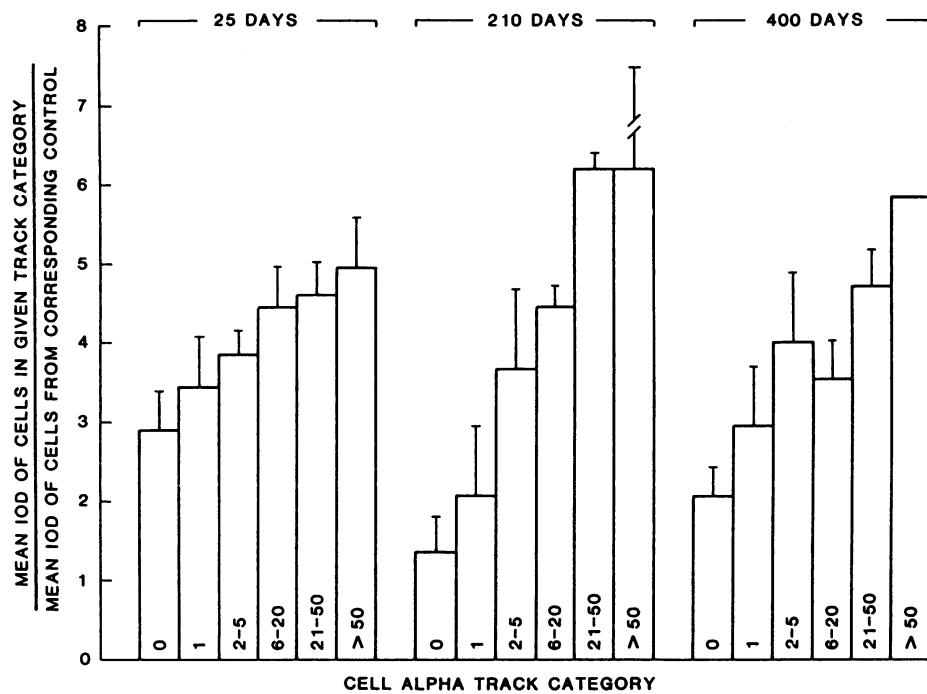
FIGURE 7.   Relationship between the β-glucuronidase activity of alveolar macrophages recovered from mice exposed to $^{239}PuO_2$ relative to levels in cells from sham-exposed controls and their associated ^{239}Pu activity at 25, 210, and 400 days after exposure.
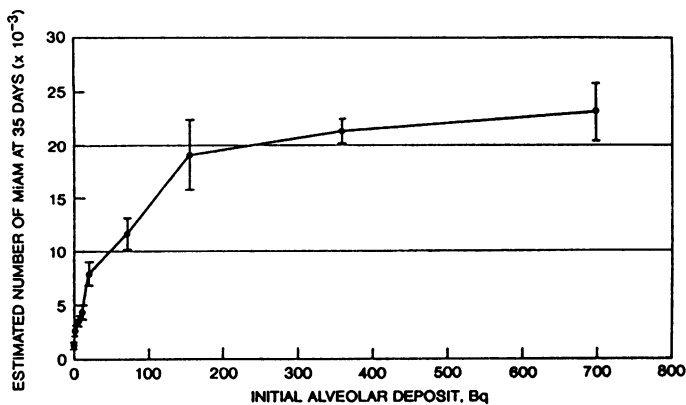

FIGURE 8.   Estimated total macrophages with micronuclei at 35 days after exposure to $^{239}PuO_2$ versus the initial alveolar deposit.
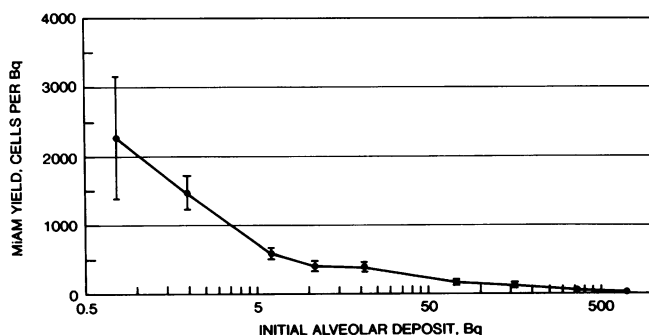

FIGURE 9.   Yield of alveolar macrophages with micronuclei per Bq initial alveolar deposit (IAD) at 35 days after exposure to $^{239}PuO_2$ versus IAD.

sus the initial alveolar deposit. It can be seen that there is a steep rise in numbers with increasing IAD at low doses, but a plateau is reached at an IAD of about 200 Bq. This corresponds to the radiation dose that results in the maximum tumor incidence in this strain of mice.

In Figure 9, the yield of MiAM per Bq IAD is plotted against IAD and shows that the efficiency with which this type of nuclear aberration is produced increases steeply at low doses. In general, the more uniform their distribution in the lung, the more carcinogenic are alpha emitters and the same applies to the yield of nuclear aberrations in AM (4). Thus, although the AM is not a tumor precursor cell, there are similarities in the dose–response relationships for the induction of micronuclei in this type of cell and for the induction of lung tumors by the same materials.

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